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Functional characterisation of glucose transport in bovine articular chondrocytes

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Abstract The adequate provision of glucose to articular chondrocytes is essential to sustain their predominantly anaerobic metabolism; glucose is also a precursor for the extracellular matrix macromolecules which these cells synthesise. Impaired glucose uptake would compromise cell function and potentially result in an imbalance of matrix synthesis and degradation, leading to osteoarthritis. We studied the glucose influx pathway into bovine articular chondrocytes using 2-deoxy-D-[³H]-glucose (DOG). Uptake occurs via an extracellular pH (pH_o)-insensitive, phloretin- and cytochalasin B-sensitive pathway, hallmarks of the GLUT family of facilitative glucose transporters, with a K_m of 0.35 ± 0.11 mM. Uptake was affected by a number of physiologically relevant factors: (1) raised hydrostatic pressure (1–30 MPa) inhibited DOG uptake by up to 30%; (2) interleukin-1 (IL-1 β) reduced uptake via an increase in transporter affinity; (3) glucosamine inhibited glucose uptake in a manner consistent with the actions of a competitive inhibitor. Given the involvement of IL-1 β in osteoarthritis and the protective role assigned to glucosamine, these findings implicate an important role for glucose delivery in chondrocyte energy production and matrix metabolism, which, therefore, may potentially affect the maintenance of cartilage integrity.

Keywords 2-Deoxy-D-glucose (DOG) · Cartilage · Phloretin · Hydrostatic pressure · IL-1 β · Glucosamine

Introduction

Chondrocytes are responsible for the maintenance of the extracellular matrix which they inhabit, which comprises

collagens and proteoglycans (PGs). Collagen gives the tissue the ability to resist tension, while PGs are trapped within the collagen fibres and, being polyanionic, attract cations together with water, creating a hydrated gel. Failure of the chondrocyte to balance synthesis and degradation of the extracellular matrix results in osteoarthritis. The homeostatic mechanisms which allow chondrocytes to withstand the physiological stresses that they experience within this matrix will play a vital role in sustaining cartilage integrity [25].

Glucose is fundamentally important in chondrocyte energy metabolism, and its uptake is central to cellular respiration and homeostasis. Given the avascularity of cartilage, the diffusion pathways between capillaries and the cells are long, such that glucose is scarce and the partial pressure of oxygen within the matrix is low [21]. Studies of chondrocyte metabolism have shown that the cells have low levels of oxygen consumption and exhibit rapid anaerobic lactate synthesis, indicating that ATP is generated predominantly by substrate-level phosphorylation in the glycolytic pathway [17]. The lactate produced within the chondrocyte leaves the cell on isoforms of the monocarboxylate transporter (MCT) family, predominantly MCT4 [11].

As the lipid bilayer of cell membranes is relatively impermeable to glucose, the latter's movement into and out of cells is achieved by means of protein carrier molecules. Although recent studies have identified at the molecular level a number of isoforms of facilitated glucose transporters (GLUTs) involved in human chondrocytes (constitutive GLUT3 and GLUT8, inducible GLUT1 and GLUT9 [18]; constitutive GLUT1, GLUT3, GLUT6, GLUT8, GLUT9, GLUT10, GLUT11 and GLUT12 [12, 16]), surprisingly few functional studies have been reported. Using an accepted congener for glucose, 2-deoxy-D-glucose (DOG) [6], here we describe the basic properties of glucose transport into bovine chondrocytes, including physiological parameters such as sensitivity to pressure and changes in extracellular pH (pH_o). In addition, we investigated the response of glucose uptake to compounds that have been suggested

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as being involved in, or as a potential treatment for, osteoarthritis, i.e. interleukin-1 (IL-1 β) and glucosamine.

Part of this study has been reported previously in abstract form [24].

Materials and methods

Materials and media

All chemicals were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. DOG was obtained from Amersham International (Bucks, UK).

The normal experimental medium HEPES-buffered saline (HBS) comprised (in mM): NaCl 145, KCl 5, MgSO₄ 1, CaCl₂ 2 and HEPES 10, with pH adjusted to 7.4 at 37 °C with NaOH. The final osmolarity of HBS was 320 mOsm. Radiolabel uptake was initiated by mixing 1 \times 10⁶ cells suspended in HBS containing 0–2.5 mM unlabelled DOG (15 min preequilibration) with an equal volume (0.5 ml) of identical HBS buffer containing the radiotracer (final activity 0.3 μ Ci ml⁻¹). Uptake was terminated by centrifugation of cells through a corn oil and dibutylphthalate mixture with a density of 1.010 (10,000 g, 10 s).

Stock solutions of inhibitors were prepared in dimethylsulphoxide (DMSO), such that the final concentration of the solvent in the cell suspensions was not greater than 1%. Glucosamine was added directly to the aqueous reaction medium without DMSO. Phloretin was used at a final concentration of 100 μ M, a value well above the reported IC₅₀ of 50 μ M for GLUT1 and 10 μ M for GLUT4, and cytochalasin B was used at a concentration of 50 μ M well above its reported IC₅₀ of 0.5 μ M for GLUT1 and 0.3 μ M for GLUT4 [8]. Where IL-1 β was used, it was added to the contents of the isolation flask before incubation and isolation. Experimental details for individual experiments are given in the figure legends.

Isolation of chondrocytes

Chondrocytes were isolated using a previously described method [23]. Briefly, feet from steers were obtained at slaughter from a local abattoir and stored at 4 °C until used (\leq 72 h). The feet were cleaned, skinned and the hooves removed before the metacarpophalangeal joints were opened under sterile conditions. Cartilage was dissected from the articular surfaces and placed in Dulbecco's modified Eagle's Medium (DMEM, osmolarity measured as 322 mOsm), supplemented with 1% v:v penicillin-streptomycin solution. The full-depth cartilage explants were incubated overnight for 18 h at 37 °C in DMEM containing collagenase (1 mg ml⁻¹). Undigested cartilage was removed by filtration, first through a cell strainer (pore size 500 μ m) and then through a Nitex filter cloth (pore size 20 μ m). The cell suspension was centrifuged (200 g; 5 min) and the pellet resuspended in fresh DMEM.

Cell viability was determined by Trypan Blue exclusion (0.5% in HBS). Trypan Blue stains only non-viable cells enabling their identification by light microscopy. Data are only presented from experiments where viability was greater than 95%. Cells were counted using a haemocytometer and resuspended in DMEM at a final concentration of approximately 1 \times 10⁶ cells ml⁻¹.

Hydrostatic pressure experiments

To assess the effect of hydrostatic pressure on DOG uptake, 0.5-ml Eppendorf tubes were adapted by drilling a 5-mm hole in the lids and affixing a latex diaphragm to the underside with epoxy glue. The reaction was initiated by mixing of radioisotope solution with cells which had been preincubated in the tube, which was then placed in a pressure cell and pressure (10–30 MPa) applied rapidly for 2.5 min. Pressure was released, and uptake stopped by centrifugation through oil as above. Previous studies in this

laboratory have shown that chondrocyte viability is unaffected by exposure to raised hydrostatic pressures [2].

Data analysis

Results are expressed as means \pm SEM, with *n* being the number of independent cell isolation batches from different animals. Experiments on cells from each isolation batch were performed in triplicate. Each isolation will produce a mixed population of chondrocytes from all depths of the explant. The significance of differences between means was determined using Student's *t*-test for unpaired samples. Analysis was performed using Prism (GraphPad Software).

Results

Uptake of DOG into chondrocytes

The rate of DOG uptake at 37 °C was time-dependent (Fig. 1), and an incubation time of 2.5 min was used subsequently to measure initial rates of uptake.

Temperature dependence of DOG uptake

To assess the temperature dependence of the initial rate of DOG uptake, the effect of varying temperature from 4 to 37 °C was measured, as shown in Fig. 2.

Concentration dependence of DOG uptake

The concentration dependence (0.1–2.5 mM) of the initial rate of DOG influx was found to be a saturable function of extracellular substrate concentration, as shown in Fig. 3. In the presence of 100 μ M phloretin or 50 μ M cytochalasin B (both well-established inhibitors of GLUT-mediated sugar transport) the data were well fitted by a linear relationship (Fig. 3), indicating inhibition of carrier-mediated uptake pathway(s) for DOG. Adding the vehicle alone (DMSO, 1%) produced a small stimulation of DOG uptake (data not shown) and so the inhibition observed cannot be ascribed to the latter's presence. *K_m*

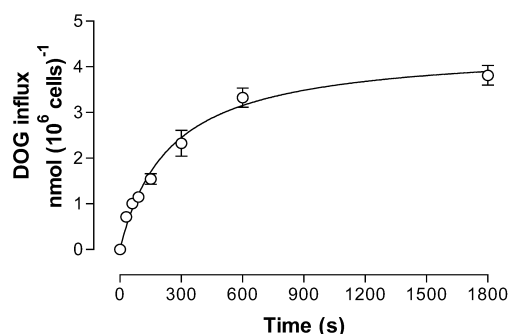


Fig. 1 Influx of 2-deoxy-D-glucose (DOG, 2.5 mM) into bovine articular chondrocytes as a function of time at 37 °C (*n*=6). Data are fitted to first-order kinetics

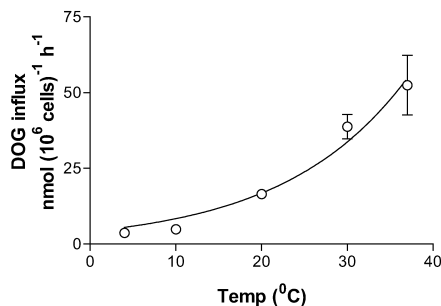


Fig. 2 Temperature dependence of DOG (2.5 mM) uptake into bovine articular chondrocytes over 2.5 min. Means \pm SEM of triplicates from one cell isolation. Q_{10} for influx was calculated to be 1.47 over the range 27–37 °C

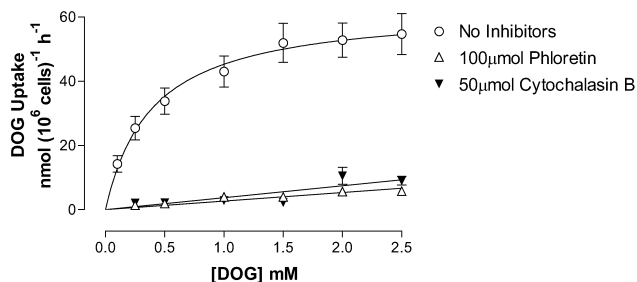


Fig. 3 Concentration dependence of DOG influx into bovine articular chondrocytes over 2.5 min and the effect of 100 μ M phloretin and 50 μ M cytochalasin B on these fluxes. ($n=9$ for control, $n=3$ for inhibitors)

and V_{\max} for carrier-mediated DOG uptake were 0.35 ± 0.11 mM and 55.7 ± 4.73 nmol $(10^6 \text{ cells})^{-1} \text{ h}^{-1}$ respectively.

Effect of hydrostatic pressure on DOG uptake

Transient hydrostatic pressures of close to 20 MPa have been recorded from pressure transducers implanted into human hip prostheses. Moreover, hydrostatic pressure has been shown to modulate glucose transport in other cell types, notably erythrocytes [13, 19]. The initial rate of DOG influx for chondrocytes subjected to a range of hydrostatic pressures (1–30 MPa) is presented in (Fig. 4) and shows a significant inhibitory effect of hydrostatic pressure.

Effect of pH on DOG uptake

In the first instance chondrocytes were exposed to a range of $[H^+]$ by resuspension in media of differing pH (pH range 6–8) at the point of initiation of flux. The initial rate of DOG uptake was not dependent on pH_o as determined by linear regression analysis ($P_{\text{non-zero}}=0.851$, data not shown).

In the second set of experiments chondrocytes were exposed the same range of $[H^+]$ by resuspension 15 min

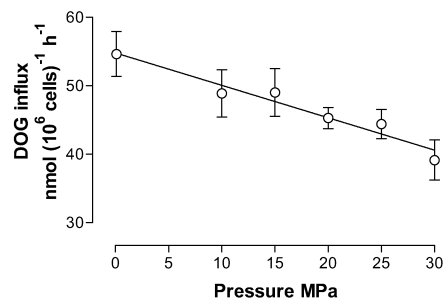
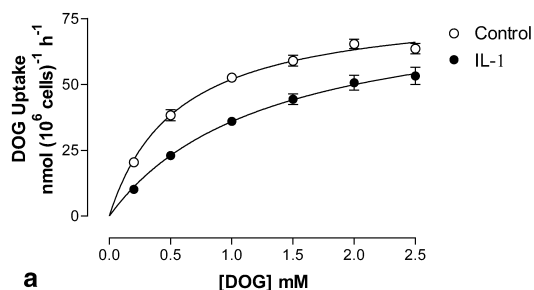
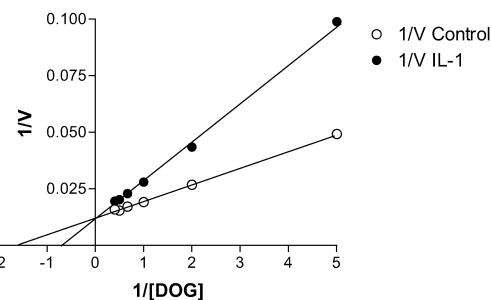


Fig. 4 Effect of hydrostatic pressure (1–30 MPa) on DOG uptake into bovine articular chondrocytes at 37 °C (2.5 mM DOG, 2.5 min, $n=3$)



a



b

Fig. 5 **A** Data showing the effect of overnight incubation in the presence of IL-1 β (0.5 ng ml $^{-1}$), on the uptake of DOG into isolated bovine articular chondrocytes at 37 °C (2.5 min, 2.5 mM DOG, $n=3$). Data are fitted with Michaelis-Menten equation. **B** Lineweaver-Burke plot of the data in **A**. For the control experiments, the x -intercept was -1.64 giving a K_m of 0.61 mM; the y -intercept was 0.01 yielding a V_{\max} of 82.6 nmol $(10^6 \text{ cells})^{-1} \text{ h}^{-1}$ ($r^2=0.953$). In the presence of IL-1 β , the x -intercept was -0.67 giving a K_m of 1.49 mM; the y -intercept was 0.0111 yielding a V_{\max} of 90.1 nmol $(10^6 \text{ cells})^{-1} \text{ h}^{-1}$ ($r^2=0.98$)

before initiation of the influx assay. The uptake of DOG was again insensitive to pH_o ($P_{\text{non-zero}}=0.955$, data not shown).

Effects of IL-1 β and glucosamine on DOG uptake

To study the effects of IL-1 β on chondrocyte DOG transport chondrocytes were incubated during overnight isolation in the absence and presence of IL-1 β (0.5 ng ml $^{-1}$). Initial rates of DOG uptake were measured for 0.25–2.5 mM DOG, and were reduced at all concentrations tested (Fig. 5A). A Lineweaver-Burke plot (Fig. 5B),

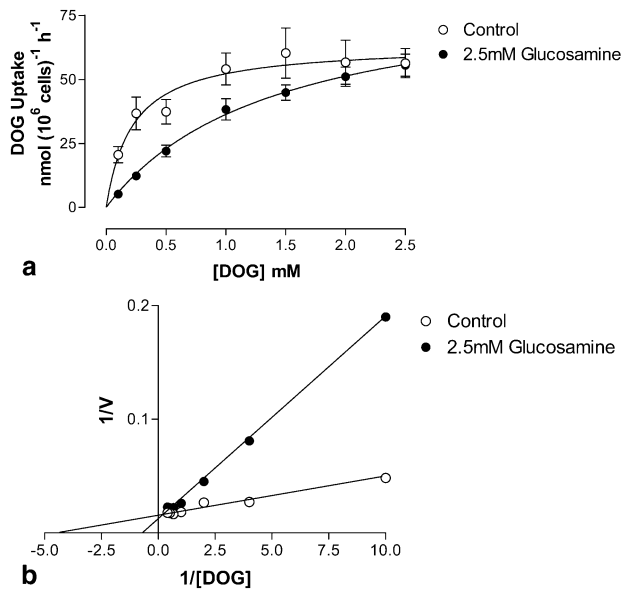


Fig. 6 **A** Effect of glucosamine (2.5 mM) on the uptake of DOG into isolated bovine articular chondrocytes at 37 °C, (2.5 min, 2.5 mM DOG, $n=3$). Data are fitted with Michaelis-Menten equation. **B** Lineweaver-Burke plot of the data in **A**. For control experiments, the x -intercept was -5.01 giving a K_m of 0.2 mM; the y -intercept was 0.016 yielding a V_{max} of 62.5 nmol (10⁶ cells)⁻¹ h⁻¹ ($r^2=0.969$). In the presence of glucosamine, the x -intercept was -0.64 giving a K_m of 1.6 mM; the y -intercept was 0.011 yielding a V_{max} of 90.9 nmol (10⁶ cells)⁻¹ h⁻¹ ($r^2=0.99$)

showed a significant reduction in the K_m of uptake, with no apparent effect on V_{max} .

To examine any effects of glucosamine on chondrocyte DOG transport, fluxes were performed for cells in the absence or presence of 2.5 mM glucosamine. At low concentrations of DOG, influx was significantly slowed, although this effect was overcome at the higher concentrations tested (Fig. 6A). The Lineweaver-Burke plot (Fig. 6B) revealed that, while glucosamine was without effect on V_{max} , K_m was increased significantly from 0.2 to 1.6 mM. Uptake of ³H-glucosamine was also measured, and found to be inhibited by glucose and phloretin (data not shown), the most likely explanation being that glucosamine is itself a GLUT substrate.

Discussion

In the present study, uptake of the glucose analogue DOG was time dependent, saturable and temperature dependent, all consistent with carrier-mediated transport. The inhibition by phloretin and cytochalasin-B is confirmatory of the expected involvement of members of the facilitative glucose transporter (GLUT) family. Kinetically, the transport has a relatively high affinity for DOG, with a K_m of approximately 0.4 mM, which is physiologically consistent with its role as a scavenger mechanism, translocating glucose from an extracellular matrix in which the substrate is scarce: the glucose concentration

surrounding chondrocytes has been estimated using microelectrodes to be approximately 1 mM (JP Urban, personal communication) due to the cells acting as a “metabolic sink”. Human chondrocytes have been shown to express a number of GLUT isoforms and thus kinetic parameters such as those measured in the experiments described here are likely to reflect contributions from a number of transporter isoforms. Nevertheless, GLUT3 is the only one of these isoforms for which there is a consensus within the literature that it is constitutively expressed. GLUT3 has the highest affinity of the GLUTs characterized to date (e.g. K_m for DOG 1.4 mM in *Xenopus* oocytes, [1]), and so GLUT3 may be predominantly responsible for the DOG uptake into bovine chondrocytes reported here.

Chondrocytes in vivo experience a wide range of pressure during routine mechanical loading, and transient increases of up to 18 MPa have been reported in human articular cartilage [7]. Here, sustained raised hydrostatic pressure during the period of uptake had a significant effect on DOG uptake into chondrocytes, with the rate of uptake decreasing as the pressure load increased. A similar finding has been reported for the erythrocyte glucose transporter GLUT1 [13, 19] and has been proposed to represent a reduction in the molar volume of the carrier protein, thereby reducing the rate at which it can undergo the conformational changes associated with substrate translocation. While it is acknowledged that static and dynamic pressures may differ in their effects, the sensitivity of glucose transport to pressure may be an important factor in determining ATP levels during joint loading.

The extracellular matrix is acidic relative to the environment experienced by most cells, partly as a result of the high fixed negative charge density which attracts free cations, including H⁺, and partly from the high rates of lactic acid produced by chondrocytes [24]. The finding that DOG transport is unaffected by increasing acidity indicates that transport will not be compromised by these acidic conditions, and agrees with earlier studies which failed to detect any pH sensitivity of the glucose carrier (see [22] for a review).

The inflammatory cytokine IL-1 β has been heavily implicated in the onset of osteoarthritis [9], initiating catabolic processes by inhibiting matrix synthesis and activating degradative enzymes. In the current study, IL-1 β reduced DOG uptake by decreasing the transporter affinity, although there was no change in V_{max} . This presumably reflects a change in the relative numbers of different GLUT isoforms expressed [16], and indeed IL-1 β induces the expression of GLUT1 and GLUT9 in human chondrocytes [18]. In contrast, Mobasheri et al. [12] have suggested that GLUT1 and GLUT9 are constitutive in chondrocytes, although this result may rather indicate that their cells had already been activated by exposure to some unphysiological stress. Although glucose transport by GLUT9 has not yet been fully characterised, GLUT1 is a low-affinity isoform, and so the increase in K_m for DOG transport seen here would be

consistent with its induction. However, the data presented here are also consistent with effects mediated by modification of the existing GLUT isoforms. In the studies by Shikhman et al. [18] and Hernvann et al. [6] IL-1 β caused an increase in measured glucose (DOG) uptake, whereas in the experiments here a modest reduction in uptake was seen under our experimental conditions (concentration range 0–2.5 mM DOG, 2.5 min incubation time). The reason for the discrepancy in the effect of IL-1 β is not immediately apparent, although the studies cited [6, 18] measured different properties. We characterised the kinetics of initial rate of DOG uptake over a range of DOG concentrations, from which we estimated values for K_m and V_{max} . The other studies offer single time-point measures of glucose uptake under conditions where transport is probably no longer occurring at its initial rate, nor is saturated with substrate. We do not consider the different findings to be mutually exclusive: the kinetics of glucose uptake will be different under the conditions employed in our study, making meaningful comparison impossible.

Glucosamine sulphate has long been regarded as having protective properties against cartilage degradation, a belief recently supported by clinical trials [15]. It is the sulphate derivative of glucosamine, a normal constituent of glycosaminoglycans in cartilage matrix and synovial fluid [10]. In the present study, glucosamine appeared to act as a competitive inhibitor for glucose uptake, with the apparent K_m for DOG increasing from 0.2 to 1.6 mM, confirmed by the reciprocal inhibition of glucosamine uptake by glucose and phloretin. It has recently been reported that glucosamine is a good substrate for a number of GLUT isoforms expressed in *Xenopus* oocytes [20], with a similar K_m for GLUT1 and GLUT4 as the natural substrate glucose, and over 20-fold higher affinity for GLUT2. In addition to glucosamine entering chondrocytes on GLUT carriers and being available for incorporation into PGs, there are increasing number of reports in the literature showing that glucosamine can inhibit the effects of IL-1 β in chondrocytes by modulating both at the IL-1 receptor and at post-receptor signalling pathway levels [3, 4, 5, 14, 16, 18].

In conclusion, uptake of DOG into bovine chondrocytes has been confirmed as being mediated by a phloretin- and cytochalasin B-sensitive mechanism, consistent with the actions of a member of the Na⁺-independent GLUT facilitated glucose transporter family. This is in agreement with recent molecular evidence for the expression of several isoforms of GLUT in human chondrocytes. DOG uptake was inhibited by physiologically relevant pressure such as might be experienced by chondrocytes during mechanical loading, and insensitive to pH_o. The catabolic agent IL-1 β decreased DOG uptake via an increase in the K_m for transport. The inhibitory effects of glucosamine are consistent with the actions of a competitive inhibitor of the DOG uptake mechanism in chondrocytes. If transported by GLUT, it could be used as a substrate for matrix synthesis and/or inhibit IL-1 β actions in the onset of osteoarthritis. The supply of

glucose for anaerobic metabolism by chondrocytes is essential for their survival and for the sustenance of matrix integrity. Understanding the effects of physiological conditions and regulators on glucose transport will give a greater understanding of chondrocyte function in healthy and osteoarthritic cartilage.

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